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A computer-assisted *in-vitro* biomaterial test for percutaneous devices using human keratinocyte cultures

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The aim of this study was to assess the suitability of a computer-assisted *in vitro* test system to evaluate candidate biomaterials used for percutaneous devices. Silicone rubber (Silastic™) and five different polymers from the Eurobiomat concerted action, polyetherurethane, polyvinylchloride with plasticizer, di-ethylhexylphthalate (PVC – DEHP), polyvinylchloride with plasticizer, tri-ethylhexyltrimellitate (PVC – TEHTM), polyethylene and polypropylene were examined with respect to their qualities to facilitate keratinocyte attachment. HaCaT-cells, a spontaneously transformed non-tumorigenic human keratinocyte cell line, were cultured on the different materials for 3 days. Cellular behaviour was examined morphologically by phase-contrast and scanning electron microscopy throughout the 72 h incubation period. For the computer-assisted measurement of the cell-covered substrate surface and subsequent statistical analysis the cells were fixed after 3 days of incubation, stained, photographed and the images then digitally transformed. Of the different polymers examined silicone rubber showed the most favourable results with respect to the quantitative analysis of the cell-covered substrate surface as well as concerning cytomorphological findings. The results of this study indicate that the *in vitro* test system described is a sensitive screening method for evaluating candidate biomaterials used for percutaneous devices in a fast and reproducible manner, thus reducing number of animal experiments.

1. Introduction

Longevity of percutaneous devices is often hampered by exit site infection, marsupialization or extrusion [1, 2]. When designing percutaneous implants it is important to select appropriate biomaterials at the skin penetration area of the implant to provide close adherence of epidermis to the implant surface resulting in a bacterial tight seal. Maintaining an intact tissue implant interface is decisive for the long-term success of percutaneous devices [2, 3, 4]. Animal experiments have mainly been used for testing percutaneous implants. Often mechanical irritation and automutilation by the animal are a limiting factor for proper evaluation of the biomaterial's potential for keratinocyte attachment [2, 4]. Histologic findings obtained from 11 continuous ambulatory peritoneal dialysis (CAPD) catheters which had been implanted in humans for periods of up to 30 months without clinical signs of infection, demonstrated that no steady state at the epidermal epithelium-implant interface was achieved. Therefore the risk of infection remained [5]. These results emphasized the need for improving the design of these catheters which were covered by Dacron velour at the skin penetration area. The choice of a more suitable biomaterial which allows for adherence of epidermal epithelium is desirable. This led to the development of an *in vitro* test system for standardized testing of biomaterials to assess their qualities facilitating keratinocyte attachment and growth.

In a previous study [6] human adult skin keratinocytes propagated under low Ca^{2+} conditions and elevated temperature (HaCaT-cells), a spontaneously transformed non-tumorigenic human keratinocyte cell line with highly preserved phenotypic differentiation characteristics of normal keratinocytes [7, 8, 9, 10], were cultured upon five different reference polymers from the EUROBIOMAT concerted action. Cellular behaviour was evaluated morphologically by phase-contrast and scanning electron microscopy

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throughout the 25 day test period and confluence of keratinocytes in relationship to the incubation period was assessed semi-quantitatively. The results demonstrated that the most significant differences between the tested materials were observed during the first three days of incubation and demonstrated the need for a technique which allows quantitative measurement of the cell-covered substrate surface, which can be used to evaluate cellular performance with respect to cell attachment, spreading and proliferation [11]. A computer-assisted method was developed for quantitative analysis of the cell-covered substrate area after 3 days of incubation. The objective of the present study was to assess the suitability of a computer assisted method for evaluating candidate biomaterials for percutaneous devices *in vitro*.

2. Experimental procedures

2.1. Materials

Six clinically applied biomaterials, silicone rubber (Silastic™) and five different polymers from the Eurobiomat multicentre research programme: polyetherurethane, polyvinylchloride with plasticizer, di-ethylhexylphthalate (PVC–DEHP), polyvinylchloride with plasticizer, tri-ethylhexyltrimellitate (PVC– TEHTM), polyethylene and polypropylene (Table I) were examined. The latter polymers have been described in detail previously [12]. The biomaterials were tested in the form of smooth films (surface roughness less than 2 μm) which were mounted between concentric polytetrafluoroethylene (PTFE) rings, using the Combi-Ring-Dish (CRD) culture chamber system developed by Noser and Limat [13, 14] (Renner Limited, Dannstadt Germany, Type B No. 30903 and Type C no. 30904), in order to facilitate culturing cells directly on the biomaterial surface.

The CRD-devices were then placed in a 24-well culture plate (Fig. 1). Polyethylene and polypropylene films were fixed at the bottom of the wells utilizing PTFE rings (Fig. 1). Silicone rubber was clamped between the concentric rings of the CRD and then vulcanized for 30 min at 120 °C. The tissue-grade polystyrene surface (Primaria®) of the multiwell plate served as control surface.

2.2. Preparation of specimens

All specimens were ultrasonically cleaned, stored in distilled water for three days, sterilized by immersion in 70% ethanol and subsequently air dried in the laminar flow hood prior to cell seeding.

2.3. Cell culture

HaCaT-cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (400 U ml^{-1}), streptomycin (50 μg ml^{-1}), glutamine (300 μg ml^{-1}), and 10% fetal calf serum (FCS) in a humidified atmosphere of 20% O_2 and 5% CO_2 and 75% air at 37 °C. HaCaT-cells of the 55th to 60th passage were seeded at a density of 1×10^5 cells/cm^2

TABLE I Different biomaterials examined

Biomaterial	Manufacturer	Thickness	Symbol
PVC–DEHP[a] Polyvinylchloride with plasticizer: Di-ethylhexylphthalate (38.4%) and stabilizer: Zn-/Ca-Stearate and epoxidized soya oil (2–3%) Batch: 29 858	Rehau Co., FRG	0.05 mm	P/D
PVC–TEHTM[a] Polyvinylchloride with plasticizer: Tri-ethylhexyltrimellitate (38.4%) and stabilizer: Zn-/Ca-Stearate and epoxidized soya oil (2–3%) Batch: 29 859	Rehau Co., FRG	0.05 mm	P/T
Polyethylene[a] Low density; Lupolene 1840 H with stabilizer Batch: MÖS 2720	Rehau Co., FRG	0.05 mm	PE
Polypropylene[a] Trespaphane® END 50 with stabilizer Batch: 1350 24 80 02	Rehau Co., FRG	0.05 mm	PP
Polyetherurethane[a] (Pellethane 2363-90 AE) medical grade; Dow Chemical (NO L 81541 10/90 TL) Batch: 910428 CENTR	Frontline Filmbläsning Co., Sweden	0.23–0.27 mm	PU
Silicone rubber Silastic®; medical grade sheetings non-reinforced, unvulcanized	Dow Corning Co., M.I., USA	0.5 mm	SIL
Control Tissue grade polystyrene Falcon Primaria®	Becton Dickinson Labware Co., NJ, USA	–	KO

[a] Refererence Materials of the Eurobiomat Concerted Action

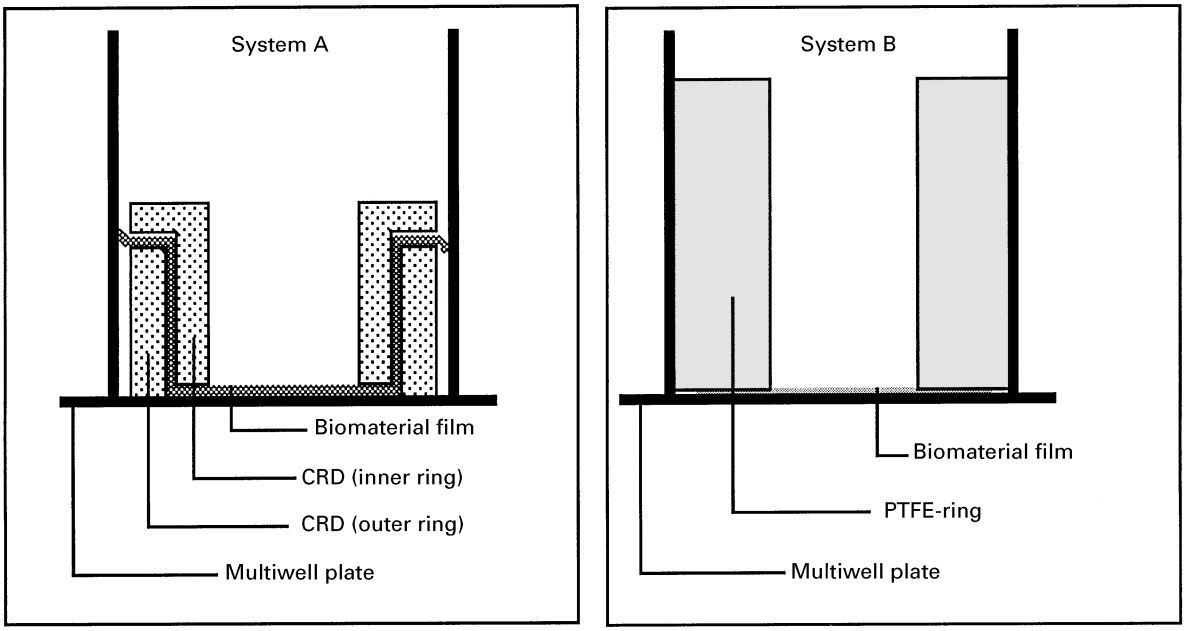

Figure 1 Schematic diagram of the devices used for application of polymer films to 24-well culture plates prior to cell culturing: system A, Combi Ring Dish (CRD); system B, PTFE-ring.

on the biomaterial films. The culture medium was changed every two to three days.

2.4. Morphological analysis

Cellular behaviour was examined morphologically by phase-contrast and scanning electron microscopy (SEM) at 3, 6, 12, 24 and 72 h of incubation. For preparation for SEM the cells cultured on the different substrates were briefly rinsed in phosphate-buffered saline (PBS) solution, pH 7.2 and fixed in 2.5% glutaraldehyde in Soerensen's phosphate buffer solution 60 mOsm, pH 7.3. for 6 h. This was followed by dehydration in graded ethanols (30, 50, 60, 70, 80, 90, 96%, and twice in absolute ethanol for 30 min each). The specimens were then soaked twice in hexamethyldisilazane (HMDS) for 10 min each and air dried for 24 h. The dried specimens were glued onto aluminium stubs, sputter-coated with gold and examined in a Cambridge Stereoscan 250 MK2 SEM.

2.5. Quantitative evaluation of the cell-covered substrate surface

For quantitative evaluation of the cell-covered substrate surface the cells were fixed after 3 days of incubation in 2.5% glutaraldehyde solution for 20 min and stained by crystal violet stain and photographed. The black and white prints were scanned and digitally transformed (Adobe Photoshop™ 38, Macintosh version) for computer-assisted measurement of the cell covered area (Image 1.35, Macintosh version) and subsequent statistical analysis (StatView 512 +™, Macintosh version). Notched-box-and-whisker-plots were utilized for graphic illustration. The differences of the groups were tested by the Kruskal–Wallis test with level of significance of $p < 0.05$. For the quantitative analysis experiments were performed five times on different days with between five and seven samples per upper (side A) and lower side (side B) of each biomaterial film being tested in each experiment to obtain sufficient data for statistical evaluation. For the silicone rubber samples only one side was examined.

3. Results

3.1. Morphological analysis

On silicone rubber spreading of HaCaT-cells occurred more rapidly and more evenly than on the other polymer films and in a similar manner, but more slowly than on the polystyrene control surface (Fig. 2). After 3 days of incubation the cell surface on silicone rubber was more smooth in appearance than on the polystyrene surface. On silicone HaCaT-cells formed mainly lamellipodia (Fig. 3). On the other biomaterial films formation of filopodia was observed more frequently (Fig. 4), indicating a reduced contact area and cells were irregularly shaped. Retarded cell spreading was encountered with these materials, greatest with polyethylene and the least with PVC–DEHP. On polypropylene there was a difference between the two sides, with cell spreading being even more retarded on side A. Cell morphology on the different biomaterials as observed after 3, 6, 12, 24 and 72 h of incubation is illustrated schematically in Fig. 2.

3.2. Quantitative evaluation of the cell-covered substrate surface

Results of the quantitative analysis of the cell-covered substrate surface are shown in Fig. 5. Statistical evaluation yielded the following data (median values). On the polystyrene control surfaces a monolayer of keratinocytes had formed with more than 99% of the test surface being covered. Of the different biomaterial

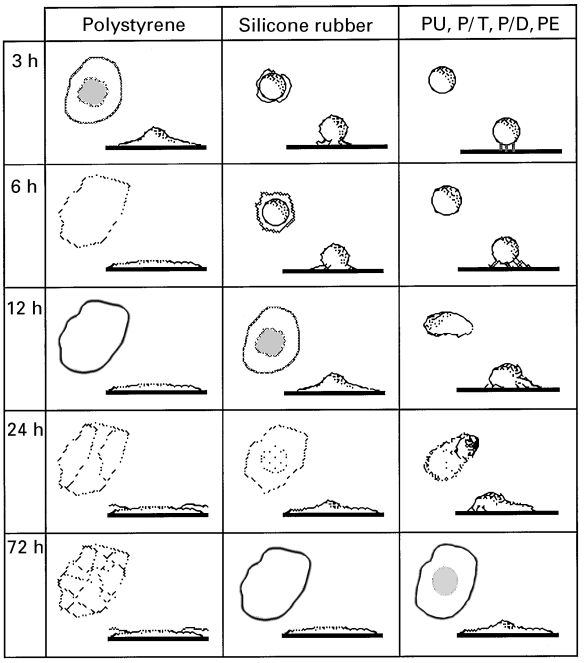

Figure 2 Schematic illustration of cell morphology on the different biomaterials tested after 3, 6, 12, 24 and 72 h of incubation (PU, polyetherurethane; P/D, PVC–DEHP; P/T, PVC–TEHTM; PE, polyethylene). On the upper left cell shapes as appearing in phase-contrast microscopy, on the lower right cross-section of spreading cells. Relatively rapid cell spreading is associated with flattened cell morphology and lamellipodia which have spread out radially.

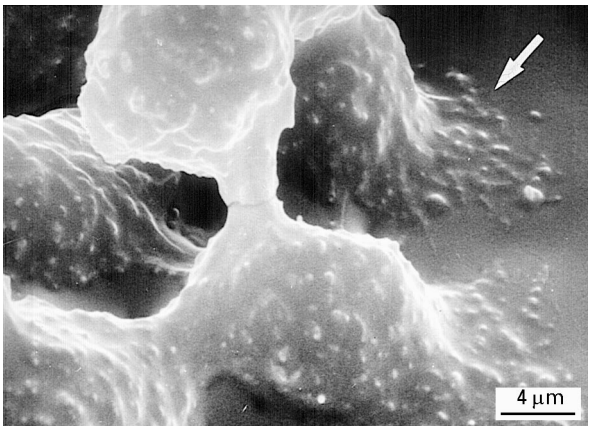

Figure 3 Scanning electron micrograph of keratinocytes cultured for 3 h on silicone rubber. Cells are spherical with lamellipodia (arrow) in contact with the silicone surface.

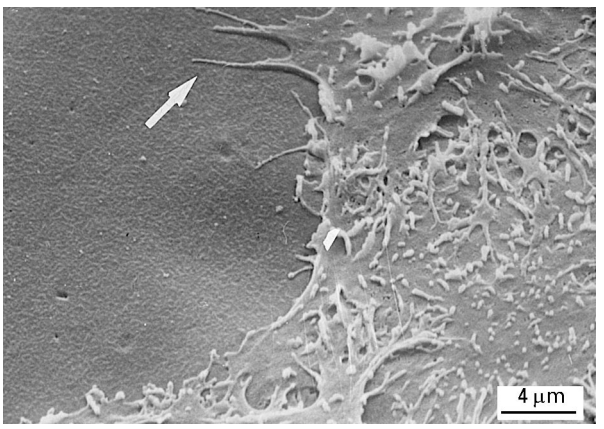

Figure 4 Scanning electron micrograph of keratinocytes cultured for 12 h on polypropylene with filopodia (arrow) extending marginally.

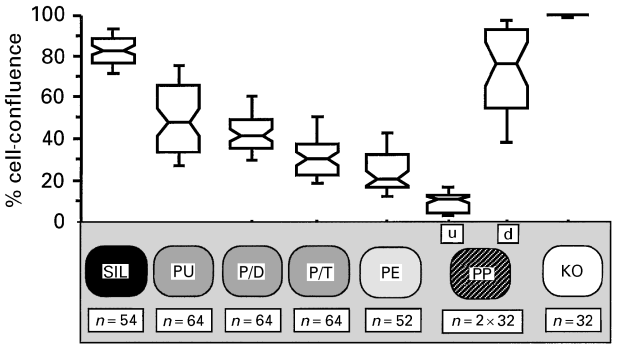

Figure 5 Boxplot diagram of cell-covered substrate surface (SIL, silicone rubber; PU, polyetherurethane; P/D, PVC–DEHP; P/T, PVC–TEHTM; PE, polyethylene, PP, polypropylene) (u = side A / upper side, d = side B / downside, KO, control).

films tested silicone rubber exhibited the highest degree of cell-covered test surface (82.9%). Compared to the other materials examined except for side B of polypropylene, there was a statistically significant ($p < 0.05$) difference. Also, values for silicone showed the least width of scattering range. Comparing the percentage of cell-covered substrate surface, there was no significant difference between polyetherurethane (47.2%) and PVC–DEHP (41.5%). However, compared to the other five substrata, the difference was statistically significant ($p < 0.05$). Median values for the percentage of cell-covered test surface were 30% for PVC–TEHTM, 20.4% for polyethylene, 10.4% for side A of polypropylene and 72.4% for side B, and 99.4% for the polystyrene control. There was a statistically significant ($p < 0.05$) difference when comparing each of these materials to the remaining six biomaterials examined. The only polymer showing a difference in percentage of cell-covered substrate surface between side A and side B of the biomaterial film was polypropylene, with the difference being statistically significant ($p < 0.05$).

4. Discussion

Morphological findings and the quantitative analysis showed corresponding results. On silicone rubber spreading of HaCaT-cells occurred faster and more evenly than on the other polymer films and in a similar manner as on the polystyrene control surface. Also silicone rubber and the polystyrene control surface exhibited the highest degree of cell-covered substrate surface. On silicone extensive formation of lamellipodia was observed, while on the other test materials filopodia predominated. Formation of lamellipodia is associated with passive cell spreading, while filopodia are indicative of the cells spreading actively [14], which requires an increase of metabolic activity, thus possibly resulting in a retarded spreading process (as observed with polyetherurethane, polyethylene, PVC–DEHP and PVC–TEHTM). Active cell spreading requires activation of cellular metabolism. This is significantly influenced by the substratum properties [15].

The surface compositions of Eurobiomat reference polymers were determined using X-ray photoelectron

spectroscopy (XPS) and static secondary mass spectrometry (SIMS) [16]. Polypropylene films exhibited significant polydimethyl siloxane (PDMS) contamination, surface oxidation and variation in composition. In fact, it appeared the surface of the polypropylene film was completely covered by a monolayer of PDMS. Contact angle analysis of polypropylene film showed that two sides of the film exhibited significant differences in contact angles [17]. Also differences in the chemical composition between both sides were found [18]. These findings might account for the differences in cellular behaviour observed with the different sides and for the considerable width of scattering range of the values for the cell-covered substrate area encountered with the lower side. The polyethylene film surface exhibited good lateral homogeneity. A small amount of a silicon contaminant such as polydimethyl siloxane (PDMS) was detected. Surface analysis of polyetherurethane indicated the presence of small amounts of PDMS and the film surface to be covered with a thin layer of the extrusion lubricant, ethylene bis-stearamide. On the PVC film samples (both PVC–TEHTM and PVC–DEHP) the dominant species present at the surface were the plasticizers and ethylene bis-stear-amide. Also PDMS was detected. The presence of low molecular weight plasticizers and stereamides on the surface of polyetherurethane and PVC samples imply that inconsistent biological results might be achieved with these materials. The steps used to prepare specimens for biological experiments may result in changes in the surface composition of these materials by removing all or part of the low molecular weight components [16]. The extended scattering range of values for the cell-covered surface area encountered with polyetherurethane may especially have to be attributed to these findings.

Since the results of toxicity test performed in mice and rabbits showed systemic toxicity for PVC–DEHP after injection of extracts and adverse local reaction after intramuscular and subcutaneous implantation, this material should not be used for percutaneous implants [19].

Results of a previous study testing different modes of sterilization (UV-light, autoclaving and immersion in 70% ethanol) demonstrated that ethanol proved to be most suitable for sterilization of the examined polymers [6].

Of the different polymer films examined silicone rubber showed the most favourable results with respect to the quantitative analysis of the cell covered surface area as well as the cytomorphological findings. Results of more recent investigations indicate that functionalization of silicon rubber consisting of carboxylate- or amino-groups [20] seems to further enhance keratinocyte attachment and growth [21]. Although cells *in vivo* adhere to and grow in a complex extracellular matrix, a vastly different surrounding from that of monolayer cultures *in vitro*, the principles of cell adhesion found *in vitro* may well be extrapolated to the *in vivo* situation at the tissue implant interface. However, further research efforts will have to follow to prove that enhanced strength of cell attachment results in increased biocompatibility *in vivo* [22].

Current research efforts include modification of the presented *in vitro* test system for evaluating different biomaterials and surface modifications using human skin fibroblast cultures (a diploid human foreskin fibroblast cell line) in addition to keratinocytes. Since percutaneous devices penetrate several tissue layers the most suitable biomaterial and surface characteristics should be selected for each cell population encountered by the implant when aiming at an optimal implant design [23].

It is concluded that the results of the present study indicate that the *in vitro* test system described is a sensitive screening method for evaluating candidate biomaterials for percutaneous devices, in a fast and reproducible manner, thus reducing number and costs of animal experiments. Of the different biomaterial films tested silicone rubber showed the most favourable results.

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References

1. A. F. VON RECUM and J. B. PARK, *CRC Crit. Rev. Bioengng* **5** (1981) 37.
2. A. F. VON RECUM, *J. Biomed. Mater. Res.* **18** (1984) 323.
3. J. A. JANSEN, J. P. C. M. VAN DER WAERDEN and K. DE GROOT, *Biomaterials* **10** (1989) 604.
4. C. GROSSE-SIESTRUP and K. AFFELD, *J. Biomed. Mater. Res.* **18** (1984) 357.
5. C. KNABE, C. GROSSE-SIESTRUP and U. GROSS, in Proceedings of the 5th Annual Conference of the Australian Society for Biomaterials, Melbourne Australia, January 1995, edited by J. A. Werkmeister, p. A 14.
6. U. NÜHLEN and C. GROSSE-SIESTRUP, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. 217.
7. N. E. FUSENIG, in "Culture of epithelial cells", edited by R. I. Freshney (Wiley-Liss, New York, 1992) p. 25.
8. P. BOUKAMP, R. T. PETRUSSEVSKA, D. BREITKREUTZ, J. HORNUNG, A. MARKHAM and N. E. FUSENING, *J. Cell Biol.* **106** (1988) 761.
9. D. BREITKREUTZ, P. BOUKAMP, H.-J. STARK and N. E. FUSENIG, in "Pharmacology and the skin", edited by N. E. Fusening (Karger, Basel, 1989) p. 8.
10. C. M. RYLE, D. BREITKREUTZ, H.-J. STARK, I. M. LEIGH, P. M. STEINERT, D. ROOP and N. E. FUSENIG, *Differentiation* **40** (1989) 42.
11. N. E. FUSENIG, in "Biology of the integument, Vol. 2, Vertebrates", edited by J. Bereiter-Hahn, A. G. Matoltsy and K. S. Richards (Springer, Berlin, 1986) p. 409.
12. W. LEMM, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. XI.
13. F. K. NOSER and A. LIMAT, *In vitro Cell Devel. Biol.* **23** (1987) 541.
14. F. GRINELL, D. J. HAYES and D. MINTER, *Exp. Cell Res.* **110** (1977) 175.

15. G. W. IRELAND, P. DOPPING-HEPENSTAL, P. JORDAN and C. O'NEILL, *J. Cell Sci. Suppl.* **8** (1987) 19.
16. D. G. CASTNER, B. D. RATNER and D. K. LEACH-SCAMPAVIA, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. 3.
17. M. COSTA, C. RINGARD, M.-M. BOISSONNADE and A. BAZKIN, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. 37.
18. H. BAUSER, H. GUNELIUS and G. HELLWIG, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. 17.
19. R. ELOY, F. CHARTON PICARD, J. BELLEVILLE and T. BOUET, in "The reference materials of the European Communities", edited by W. Lemm (Kluwer, Dordrecht, the Netherlands, 1992) p. 237.
20. R. SIEGEL, Offenlegungsschrift DE 42 16 271 A 1, German Patent Office 1993.
21. S. SCHÜRNER-RITTER, D. KNEIDING and C. GROSSE-SIESTRUP, *Int. J. Artif. Organs* **18** (1995) 478.
22. A. F. VON RECUM and T. G. van KOOTEN, *J. Biomater. Sci., Polym. Ed.* **7** (1995) 181.
23. D. M. BRUNETTE, *Int. J. Oral Maxillofac. Implants* **3** (1988) 231.

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